PRESERVATION OF FUNGI

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SUMMARY: Dehydration of cells, necessary to avoid intra-cellular crystallization during freezing, is regulated through the cooling rate and depends on the size of the cell and the thickness of the cell-wall. Saccharides prevent phase transitions in the membrane from the liquid-crystalline to the gel-phase during dehydration. Proteins are protected against denaturation during slow cooling by saccharides and amino-acids. During freeze-drying, cells are dehydrated in the cooling step preceding drying; during drying the protectant is converted in a glass. Cryoprotectants such as glycerol, dimethylsulfoxide, 1.2 propane-diol, ethylene-glycol, ethanol, methanol, polyethylene-glycol and several butane-diols serve to avoid extra - and intracellular crystallization, but most of them are toxic. Revival in 1.2 M sucrose is recommended.

KEYWORDS: fungi, cryopreservation, lyophilization

Fungi can be preserved by various methods: on agar, under mineral oil, at ultralow-temperature and lyophilized. The latter two methods (long-term preservation) are the least laborious because metabolism is arrested and negative effects of degeneration and infection are minimized.

Cooling

The cooling rate is a critical parameter in both long-term preservation methods. When cells are cooled rapidly, intra-cellular ice-crystals are produced, which is often lethal. Production of intracellular crystals can be avoided by slow cooling (Morris, 1981). At slow cooling rates the bulk of extracellular water crystallizes in pure ice-crystals leaving a highly concentrated eutectic solution. Osmotic equilibrium between this solution and the cell is maintained by cell shrinkage. Consequently the cytoplasm becomes increasingly concentrated resulting in a depression of the freezing temperature (Fig. 1). At the optimal cooling rate cells are frozen at a rate that intracellular freezing is just avoided completely. This cooling rate depends on the size of the cell and the thickness of the cell-wall and can be monitored with a cryo-microscope (Mazur, 1984; Tan *et al.*, 1994).

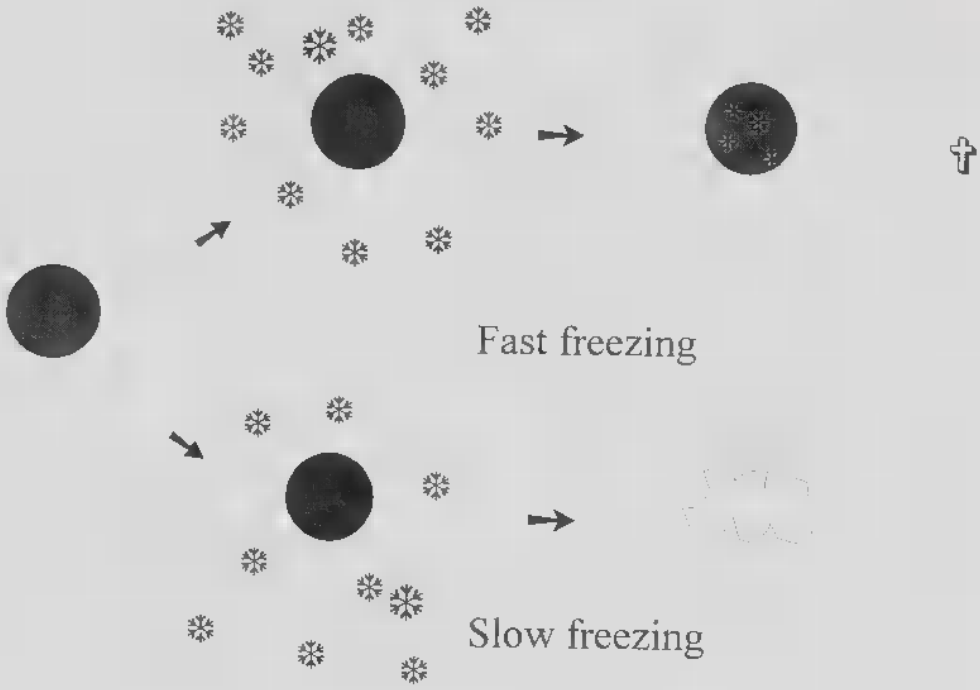

Fig. 1. — Influence of the cooling rate on cells.

Freeze-drying

Lyoprotectant:

During freeze-drying, cells are suspended in a lyoprotectant. A lyoprotectant includes a macromolecule, which serves as a bulking agent, and a saccharide. In a fluid (liquid-crystalline) membrane, water is hydrogen-bonded to the phospholipid head groups, yielding space for the fatty-acid acyl chains to be mobile. During desiccation a phase transition occurs in the membrane from the liquid-crystalline to the gel phase, which causes leakage of the membrane and therefore will result in cell damage. Saccharides protect membranes during freezing and drying (Crowe *et al.*, 1984, 1987, 1990) against this transition by hydrogen-bonding to the phospholipid head groups thus replacing water (Crowe *et al.*, 1985a). This interaction increases head group spacing, resulting in a lower transition temperature of the phospholipids (Crowe *et al.*, 1985b) (Fig.2). Disaccharides are found to be optimal, particularly trehalose (Fig. 3) (Crowe *et al.*, 1984; Tan *et al.*, 1995). Trehalose is produced in spores and conidia of yeasts and fungi to protect their membranes and proteins at the low moisture content present in these propagules (Thevelein, 1984; Wiemken, 1990).

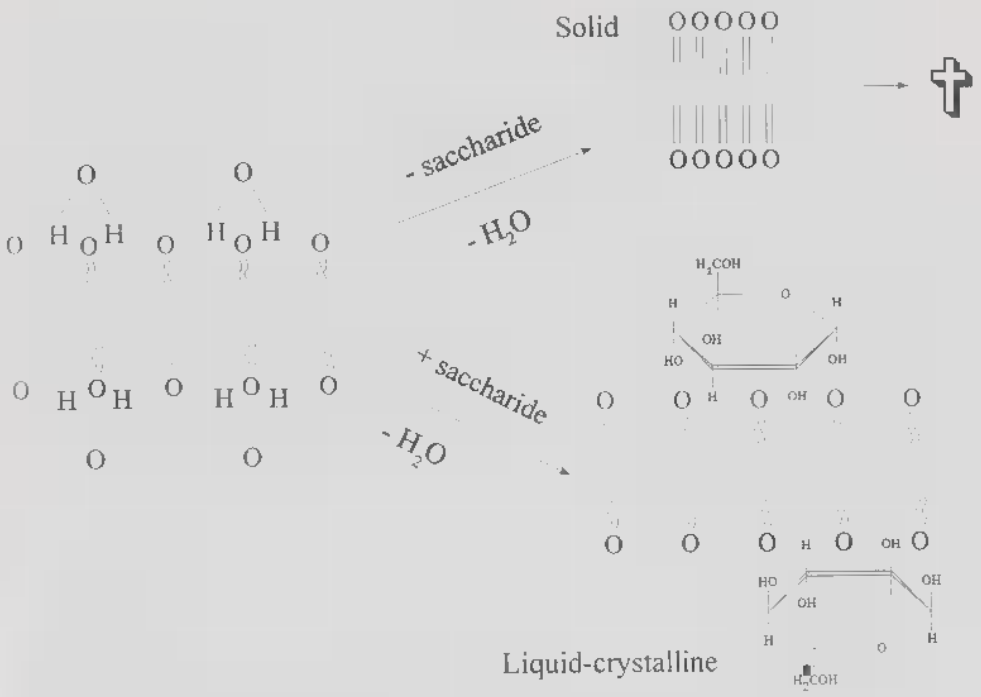

Fig. 2. — Prevention of phase-transition of the membrane during dehydration by saccharides.

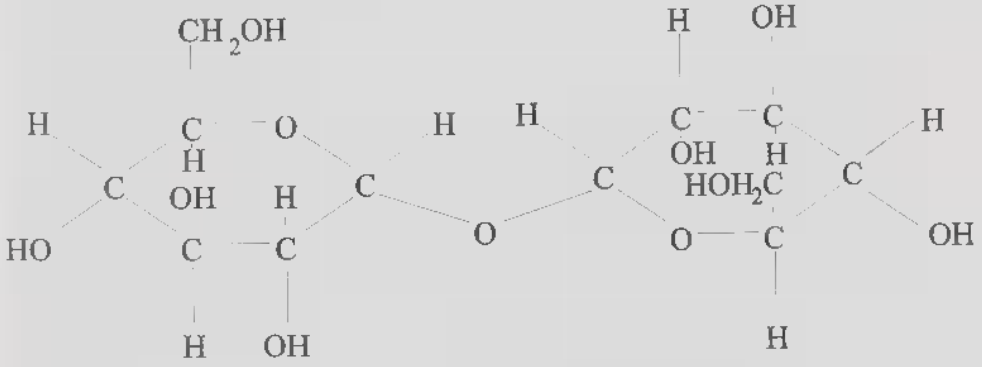

Fig. 3. — Structure formula of trehalose.

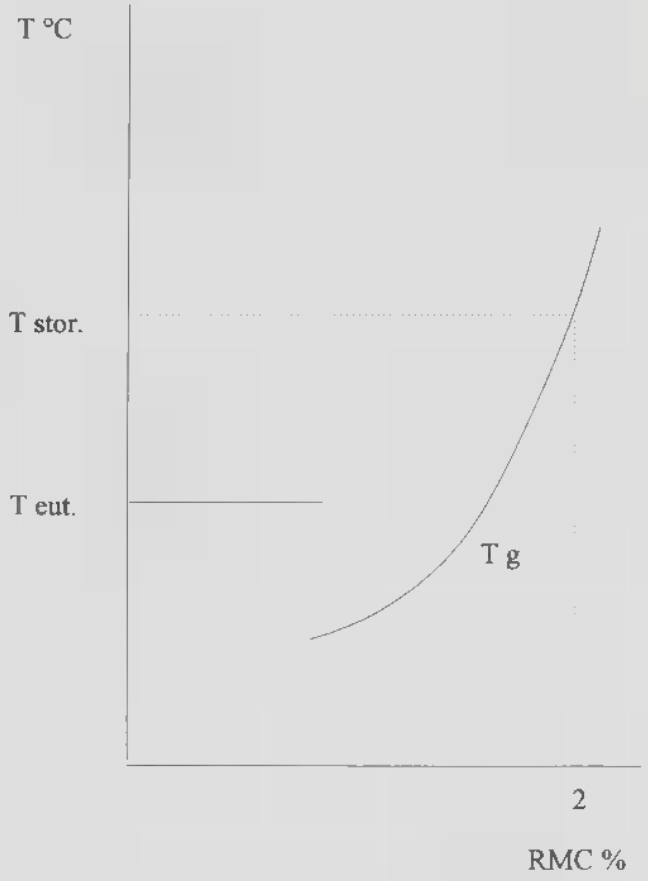

Fig. 4. — Phase-diagram of solution during freeze-drying.

As a result of freeze-concentration during slow cooling, proteins unfold and hence denaturate. They can be protected against this denaturation by saccharides and amino-acids. Proteins are stabilized because both type of compounds are preferentially excluded (Arakawa & Timasheff, 1982; Back *et al.*, 1979; Carpenter & Crowe, 1988) from the surface of the protein in aqueous solution. They repel the hydrophobic parts of the amino acid chains, thus preventing unfolding of the protein. Moreover, hydrogen bonding between the saccharide and the protein in the final stages of desiccation is required for stabilization of the dried proteins (Carpenter & Crowe, 1988; Carpenter *et al.*, 1991).

Freeze-drying protocol:

In the cooling step preceding drying the bulk of extracellular water crystallizes and the cells are dehydrated. After cooling the dehydrated cells, surrounded by the highly

viscose lyoprotectant are embedded in ice-crystals. At the temperature of the primary drying phase, viscosity of the lyoprotectant is so high that it is a glass. A glass is a liquid in which the molecules are immobilized (Franks, 1990). In the primary drying phase the ice-crystals evaporate, leaving a glass interwoven with channels. During secondary drying water evaporates through these channels from the glass, making it even more viscose. Because the viscosity increases, the temperature at which the glass is stable increases. By slowly raising the temperature, evaporation of water from the glass is enhanced. Finally so much water is evaporated that the protectant is a stable glass at room temperature (Fig.4). A glass is an ideal formulation to store dehydrated organisms because they are protected against outside enzyme and chemical activity and the molecules of the lyoprotectant are arranged in an unordered structure, allowing binding to the membranes and the proteins. The temperature-regime, that must be applied to prevent collapse of the glass during freeze-drying, can be established with a freeze-drying microscope.

Freeze-dried organisms can be stored successfully below the glass-transition temperature (Tg) (Franks, 1990). Tg is the temperature at which the glass melts during warming. Above Tg, water mobility increases and consequently the product deteriorates. The Tg is determined by the composition of the protectant and the residual moisture content and can be estimated by differential scanning calorimetry (DSC) (Hatley, 1990).

Cryopreservation

Freeze-drying has two advantages over cryopreservation. No special requirements are needed to store the product and its despatch does not need cooling facilities. However, viability is much higher for cryopreservation and sterile mycelia can generally not be freeze-dried. During cryopreservation, cells are suspended in a cryoprotectant. Cryoprotectants have a high glass-forming tendency and the glasses produced are rather stable (Mehl & Boutron, 1987; Boutron *et al.*, 1986). They partly penetrate the cells where they prevent or reduce growth of intra-cellular ice-crystals. Because not all the protectant penetrates the cell, they stimulate cell shrinkage and hence lower the freezing temperature of the cellular contents. Finally they reduce growth of ice-crystals in the medium. Commonly used cryoprotectants are glycerol, dimethylsulfoxide, 1.2 propane-diol, ethylene-glycol, ethanol or methanol. Other solutions having a high glass-forming tendency are poly-ethylene-glycol, 1.2 butane-diol, 1.3 butane-diol and 2.3 butane-diol (Mehl & Boutron, 1987; Boutron *et al.*, 1986) but they are quite toxic. It is advisable to estimate the time required for permeation of the cryoprotectant into the cell by cryomicroscopy, and in relation to this and the size of the cell and the thickness of the cell-wall, the optimal cooling rate. Moreover, since most cryoprotectants are more or less deleterious, toxicity must be established to determine the period and temperature of handling prior to storage at ultra-low temperature. Organisms can best be stored below -135°C to completely stop the growth of ice-crystals (Morris, 1981). When stored at a higher temperature, the product must be kept below the Tg of the protectant.

Revival

Revival is an important issue in both long-term preservation methods because sublethal damage on membranes and proteins can never be avoided completely. Cells can be revived in water or transferred immediately onto the suitable agar-medium. However, when survival rates are low, it is recommended to revive cells in 1.2 M sucrose to dilute the toxic protectant and to stimulate efflux of the protectant from the cell while minimizing

osmotic expansion (Tan & Stalpers, 1996). Optionally amino-acids may be added to the revival medium to repair denaturated proteins and to restore the energy charge.

Storage under mineral oil

When neither technicians nor equipment are available for maintenance of fungal cultures, storage under mineral oil is a cheap and less elaborative alternative (Fennel, 1960). Cultures, growing on an agar slant are covered by paraffine oil (Paraffinum perliquidum 60-80 (mPa.s = 1 cP), Brocacef B.V., Maarssen, The Netherlands). Oxygen entrance to the cultures, and hence metabolism is retarded to approximately 10%. When stored at 10°C and at a relative humidity of 70%, ascomycetes, basidiomycetes and zygomycetes have to be transferred only once in ten years. Oomycetes must be transferred every two years when stored under these conditions.

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